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Effect of biological sex and short-term high-fat diet on cellular proliferation, ribosomal biogenesis, and targeted protein abundance in murine articular cartilage

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ABSTRACT

Objective: To identify factors contributing to sex-differences in OA risk by evaluating the short-term effect of high-fat (HF) diet on sex-specific changes in cartilage cell proliferation, ribosomal biogenesis, and targeted extracellular and cellular protein abundance.
Materials and methods: Knee cartilage was harvested to the subchondral bone from 20-week-old female and male C57BL/6J mice fed a low-fat or HF diet for 4 weeks and labeled with deuterium oxide for 1, 3, 5, 7, 15, or 21 days. Deuterium enrichment was quantified in isolated DNA and RNA to measure cell proliferation and ribosomal biogenesis, respectively. Protein concentration was measured using targeted high resolution accurate mass spectrometry.
Results: HF diet increased the maximal deuterium incorporation into DNA from approximately 40 to 50%, albeit at a slower rate. These findings, which were magnified in female versus male mice, indicate a greater number of proliferating cells with longer half-lives under HF diet conditions. HF diet caused distinct sex-dependent effects on deuterium incorporation into RNA, increasing the fraction of ribosomes undergoing biogenesis in male mice and doubling the rate of ribosome biogenesis in female mice. HF diet altered cartilage protein abundance similarly in both sexes, except for matrilin-3, which was more abundant in HF versus LF conditions in female mice only. Overall, HF diet treatment had a stronger effect than sex on cartilage protein abundance, with most changes involving extracellular matrix and matrix-associated proteins.
Conclusions: Short-term HF diet broadly altered cartilage matrix protein abundance, while sex-dependent effects primarily involved differences in cell proliferation and ribosomal biogenesis.

1. Introduction

A central feature of osteoarthritis (OA) is the loss of articular cartilage due to an imbalance in cartilage anabolic and catabolic processes [1]. At late stages of OA, this imbalance involves reduced anabolic and enhanced catabolic processes associated with numerous cellular changes, including chondrocyte apoptosis, senescence, ribosomal dysfunction, endoplasmic reticulum stress, and metabolic dysfunction [2–5]. However, at early stages of OA, several anabolic activities are elevated, such as rates of chondrocyte proliferation and type II collagen synthesis [6,7]. A deeper understanding of the relationship between chondrocyte turnover and cartilage biosynthetic activity is needed to characterize cell-intrinsic changes during the development of OA.

We recently described a stable-isotope method using *in vivo* deuterium oxide labeling and mass spectrometry to measure cell proliferation, ribosomal biogenesis, and protein remodeling in murine articular

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cartilage [8]. We observed an age-associated reduction in cellular protein content, cartilage extracellular matrix protein content, and extracellular matrix protein synthesis rate without detectable changes in cellular proliferation or ribosomal biogenesis. An unexpected finding was that cellular proliferation and ribosomal biogenesis were already fully turned over by our earliest labeling timepoint of 15 days. Therefore, to characterize these rapid cellular processes, deuterium oxide labeling must be conducted over a shorter period.

The goal of this study was to use short-term deuterium oxide labeling and mass spectrometry to evaluate the sex-dependent effect of 4 weeks high-fat (HF) diet on cartilage cell proliferation, ribosomal biogenesis, matrix protein content. From a clinical perspective, obesity is one of the most important contributors to the rise in OA prevalence [9], and HF diet-induced obesity is an established preclinical model of knee OA in C57BL/6J mice [10–13]. In previous studies, we showed that 20 weeks of HF diet caused low-grade OA pathology and altered chondrocyte metabolism and proteostasis [14–16]. Here, we tested if a HF diet would alter cartilage homeostasis with just 4 weeks of treatment. In addition, we tested for sex differences in the effect of HF diet on cartilage homeostasis. Biological sex is a key OA risk factor [17–19], and recent population-based studies indicate that obesity has a greater impact on the incidence of knee OA and total knee replacement in women versus men [20,21]. We hypothesized that short-term HF diet treatment would induce sex-specific differences in cartilage cell proliferation, ribosomal biogenesis, and matrix protein content.

2. Materials and methods

2.1. Study design and animal experimentation

All experiments were conducted in accordance with protocols approved by the AAALAC-accredited Institutional Animal Care and Use Committee at the Oklahoma Medical Research Foundation (OMRF). 12–13-week-old male (n = 36) and female (n = 36) C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in the OMRF vivarium under veterinary care. Mice were group housed (3 animals/cage) in ventilated cages in a temperature-controlled room maintained at 22 ± 3 °C on 14 h:10 h light/dark cycles with *ad libitum* access to chow and water. At 16 weeks of age, mouse cages were randomly assigned to low-fat (LF; Research Diets D12450Ji; 10 kcal% Fat) or high-fat (HF; Research Diets D12492i; 60 kcal% Fat) diet for 4 weeks. Mice were given an initial intraperitoneal bolus injection of 99% deuterium oxide (D_2O), followed by *ad libitum* access to drinking water enriched with 8% D_2O. The initiation of D_2O labeling to a cage was staggered so that mice were labeled 1, 3, 5, 7, 15, or 21 days (n = 3 per diet, sex, and labeling duration) prior to euthanasia. Thus, the duration of diet treatment and age at the time of euthanasia were the same for all animals despite different labeling periods (Fig. 1A). Cartilage, liver, skeletal muscles, and blood were collected after euthanasia by exsanguination via cardiac puncture under isoflurane anesthesia. Blood was centrifuged at 2,000g for 10 min, and serum was aliquoted and frozen at −80 °C until further analysis. Tibial and femoral articular cartilage was immediately extracted and stored as previously described [8].

2.2. RNA/DNA/protein isolation

Total DNA, RNA and protein were isolated from articular cartilage, as previously described [8]. Briefly, frozen cartilage samples were homogenized to a frozen powder using a TissueLyser (Qiagen). TRIzol® Reagent (Thermo Fisher, Rockford, IL, USA) was added to the powdered frozen cartilage, and samples were centrifuged at 16,000g for 10 min at 4 °C. The supernatant was discarded, and 160 μL of chloroform was added. The mixture was vigorously shaken and then centrifuged at 12,000g for 15 min at 4 °C. The solution separated into three phases: a transparent RNA-rich aqueous phase, a protein-rich TRIzol phase, and a DNA-rich interphase and pellet. The steps for isolating RNA, protein, and DNA were performed as previously described [8].

2.3. Cell proliferation and ribosomal biogenesis

Cell proliferation and ribosomal biogenesis were determined by measuring the incorporation of D_2O in isolated DNA and RNA according to our previously published method [8]. Briefly, DNA and RNA were hydrolyzed, derivatized, and analyzed on an Agilent 8890 GC coupled to an Agilent 7010B MS. Data were analyzed using MassHunter software. All analyses were corrected for natural isotope abundance with an unenriched pentafluorobenzyl triacetyl purine ribose/deoxyribose derivative standard. To determine body water deuterium enrichment, plasma samples were prepared for analysis on a liquid water isotope analyzer (Los Gatos Research, Los Gatos, CA, USA) using 0%–12% deuterium standards.

The precursor enrichment of ribose and deoxyribose was calculated using mass isotopomer distribution analysis to determine equilibration with the body water pool [22]. The deuterium enrichments of both DNA or RNA (product) and the precursor were used to calculate fraction new. Fraction new = $E_{product}/E_{precursor}$, where the $E_{product}$ is the enrichment (E) of DNA or RNA and $E_{precursor}$ is the DNA or RNA enrichment calculated from equilibration of the body water pool. The rate of synthesis was calculated from the plot of fraction new versus time using a one-phase association (k, 1/day) (GraphPad Prism 10). Plotting the fraction of new DNA or RNA synthesis versus time allows for the determination of plateau fraction new (p). When p is <100%, the remainder (1-p) represents the fraction of the DNA or RNA pool that did not renew [23]. Comparing the fraction of the DNA or RNA pool that actively renews versus that which did not is biologically important as it provides insight into the potential to remodel. Traditional synthesis rate calculations that adjust for the total DNA or RNA pool by multiplying k times p and their combined variance underestimates the turnover rate of the active pool [24] and is provided for comparison. Cellular half-lives were calculated from the equation $\tau_{1/2} = \ln(2)/k$, where k is a cell proliferation rate for either active or total cell pool.

2.4. Protein quantitation by targeted proteomics

Protein samples were prepared as previously described [8,25]. Briefly, 50 μg of total protein was processed with an internal standard of 8 pmol BSA added to the sample in 1% SDS. After desalting by precipitation in 1 mL of acetone overnight at −20 °C, protein was solubilized in 50 μL Laemmli sample buffer and 20 μg protein was run in a 12.5% SDS-Page gel (BioRad Criterion system). Gels were fixed and stained with Coomassie blue (GelCode blue, Pierce Chemical Company). Samples were cut from the gel as the entire lane, divided into smaller pieces, and washed to remove the Coomassie blue. Samples were then reduced in 10 mg/mL DTT, alkylated in 35 mg/mL iodoacetamide, and digested overnight with 1 μg trypsin per sample in 200 μL 10 mM ammonium bicarb. The mixture of peptides was extracted from the gel, evaporated to dryness in a SpeedVac, and reconstituted in 150 μL 1% acetic acid (v/v) for LC-tandem MS analysis.

Protein concentrations were determined by LC-high resolution MS using a QEx Plus hybrid quadrupole-orbitrap mass spectrometry system (ThermoScientific), a splitless nanoflow HPLC system with autoinjector (ThermoScientific), and a 10 cm C18 column (Phenomenex Aeris 3.6 μm Peptide XB-C18 100A) packed in a fused silica electrospray tip (New Objective). 5 μL sample volumes were injected and loaded onto the column at 1.5 μL/min with 0.1% formic acid. The column was eluted at 150 nL/min with a linear gradient of CH_3CN in water with 0.1% formic acid (2% CH_3CN to 65% CH_3CN in 60 min). The orbitrap mass spectrometer acquired full scan mass spectra with a m/z resolution of 280,000. Ion source settings included a spray voltage of 1.5 kV, ion transfer tube temperature of 300 °C, and positive ions mode.

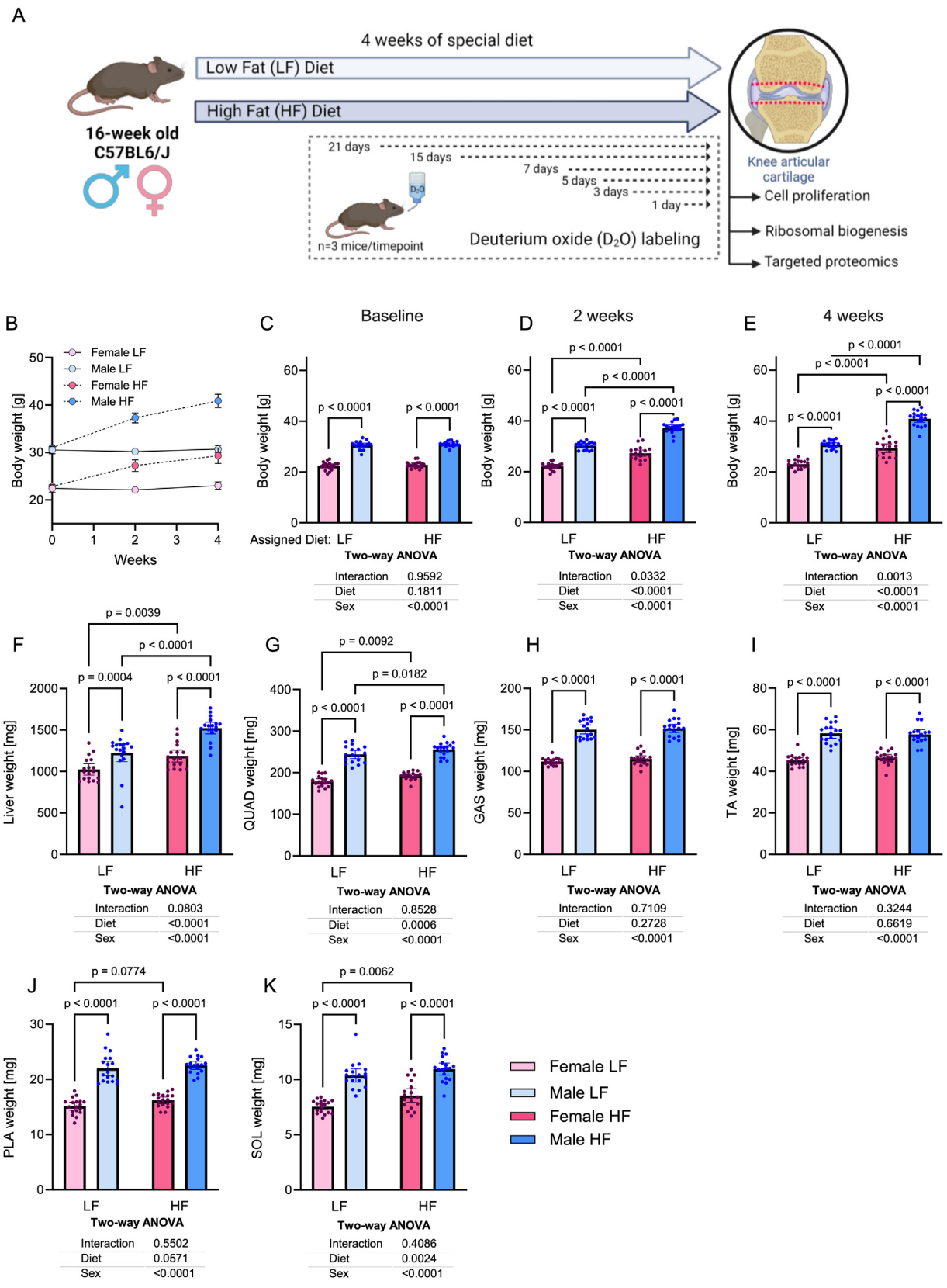

Fig. 1. Study design and animal characteristics. (A) Overview of experimental design and deuterium oxide (D_2O) label duration for each animal cohort. (B) Body weights of female and male C57BL6/J mice at (C) 0, (D) 2 and (E) 4 weeks of LF and HF diet treatment. Following 4 weeks of diet treatment, organ weights for (F) liver and hindlimb skeletal muscles, including (G) quadriceps (QUAD), (H) gastrocnemius (GAS), (I) tibialis anterior (TA), (J) plantaris (PLA), and (K) soleus (SOL). Each point represents a single animal. Data presented as mean ± 95%CI. Effects of diet and sex were evaluated using Two-way ANOVA. Fisher LSD test was used for post-hoc multiple comparisons (p-values shown for $p < 0.10$). n = 18/per group.

A total of 44 pre-validated ECM, ECM-associated, and cellular proteins were measured in these experiments [8]. The targeted high-resolution accurate mass (HRAM) analyses were performed in Skyline Software [26] using at least two unique peptides from each protein (http://www.peptideatlas.org/). The response for each protein was calculated as the total integrated chromatographic peak areas for all peptides monitored for that protein. Targeted protein concentration was calculated as the geometric mean of all unique peptides [8], normalized to the BSA internal standard.

2.5. Statistical analyses

We used a sample size of 3 animals per time point based on our prior studies using D_2O labeling for measuring cell proliferation, ribosomal biogenesis and protein synthesis in cartilage and other tissues [8,23,27]. Importantly, the calculated DNA and RNA synthesis rate and error are determined by the goodness of fit of the line to data from all mice (n = 18, 3 mice/timepoint x 6 time points). Note that n = 15 for RNA because 21-day timepoint samples were not available due to a sample processing error. Importantly, deuterium incorporation into newly synthesized RNA

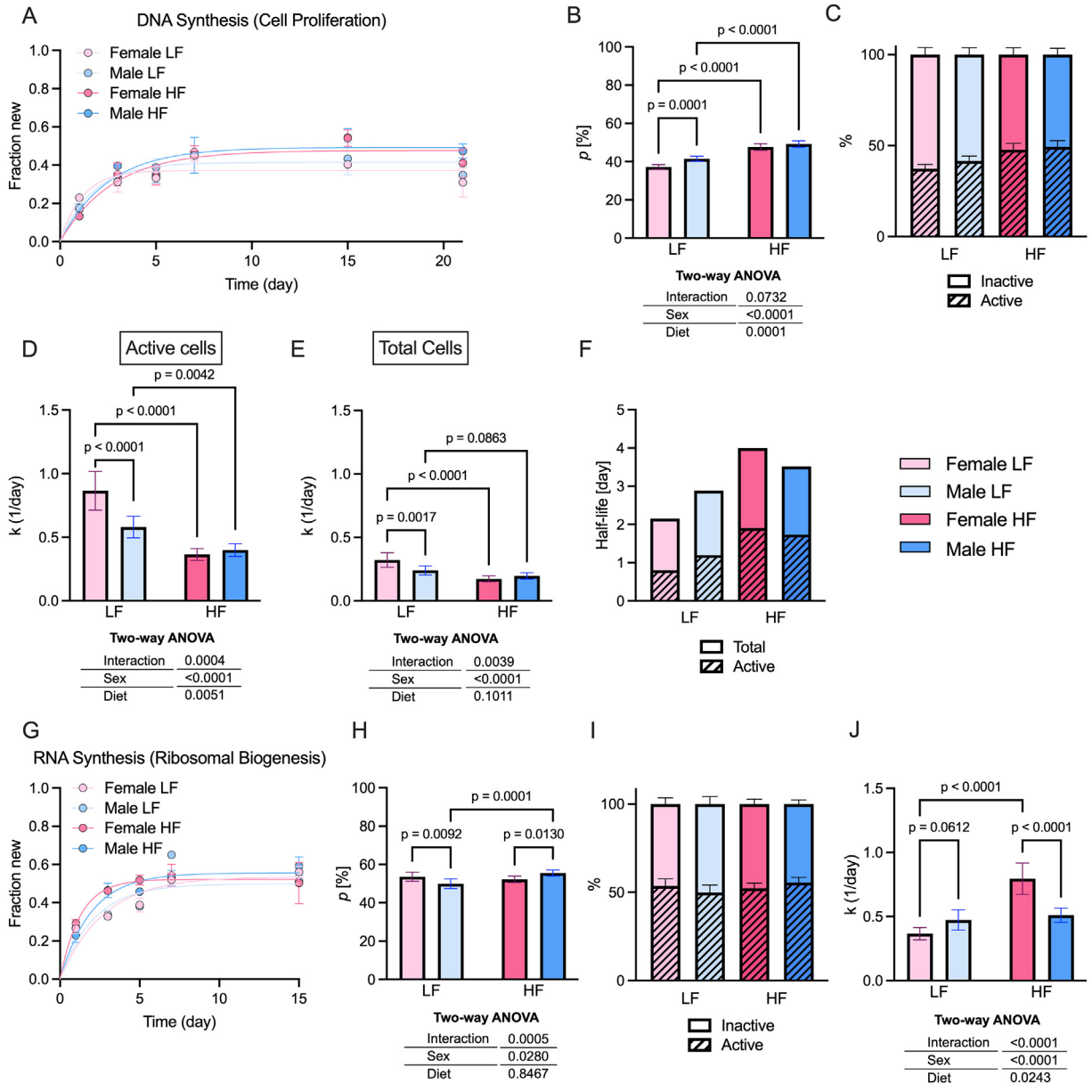

Fig. 2. Effect of 4-week LF and HF diet treatment on cell proliferation and ribosomal biogenesis in knee articular cartilage from female and male mice. (A) Fraction of newly synthesized DNA in extracted knee articular cartilage samples based on deuterium incorporation following 1-, 3-, 5-, 7-, 15- and 21-days of labeling in LF and HF diet-treated female and male mice. (B) Plateau values (*p*) of deuterium-labeled DNA relative to the total DNA pool. (C) Representation of the relative proportion of active and inactive pool sizes of proliferating cells, as determined from *p*. (D) DNA synthesis rates (*k*) specific to the pool of actively proliferating cells, which is proportional to the rate of cell proliferation. (E) DNA synthesis rates (*k*) for the total cell pool, reflecting the overall average rate of cell proliferation. (F) Cell half-life calculations derived from synthesis rates for the active and total cell pools. (G) Fraction of newly synthesized RNA in extracted knee articular cartilage samples based on deuterium incorporation following 1-, 3-, 5-, 7-, 15- and 21-days of labeling in LF and HF diet-treated female and male mice. (H) Plateau values (*p*) of deuterium-labeled RNA relative to the total RNA pool. (I) Representation of the relative proportion of ribosomes undergoing biogenesis relative to the total ribosome pool, as determined from *p*. (J) RNA synthesis rates (*k*), indicating rates of ribosomal biogenesis. Data are mean ± 95% CI. Effects of diet and sex were evaluated using Two-way ANOVA. Fisher LSD test was used for post-hoc multiple comparisons ($p < 0.10$ shown). DNA samples sizes: n = 17 to 18 per group. RNA sample sizes: n = 13 to 15 per group (21-day labeling unavailable due to processing error).

reached a plateau by day 7. Therefore, loss of 21-day RNA samples has a negligible impact on RNA synthesis measurements. The effects of diet and sex on organ weights, parameters of cell proliferation and ribosomal biogenesis, and protein concentrations were evaluated using two-way ANOVA. Tests showing a significant effect of diet or sex ($p < 0.05$) were followed by Fisher LSD post-hoc tests to identify specific group differences. Some samples did not yield sufficient DNA, RNA, or protein for analysis, as indicated in figure legends. All values were reported as mean ± 95% CI. GraphPad Prism version 10.1.1 (GraphPad Software, San Diego, California, USA) was used for calculations and data presentation.

3. Results

3.1. Effects of sex and diet on body and organ weights

Male mice were ~30% heavier at baseline than female mice (Fig. 1B-C). Body weights were not different between mice of the same sex randomly assigned to HF or LF diets before treatment (i.e., "0 weeks"), and body weights of mice assigned to LF diet did not change throughout the 4-wk treatment. Conversely, after just 2 weeks of HF diet, male and female mice increased body weight by ~20% compared to respective baselines (Fig. 1D). 4-weeks of HF diet increased body weights 32% for male mice and 28% for female mice compared to baseline values (Fig. 1E). As expected, liver and skeletal muscles weights were greater in male versus female mice (Fig. 1F–K). However, an effect of HF diet on organ weight was only observed for liver (Fig. 1F) and two skeletal muscles, quadriceps (Fig. 1G) and soleus (Fig. 1K), where weights were greater with HF versus LF diet.

3.2. Sex-dependent effect of HF diet on chondrocyte proliferation and ribosomal biogenesis

Newly synthesized DNA reached a plateau within 7 days of labeling (Fig. 2A). Both HF diet and sex significantly altered the plateau value (p), which represents the fraction of the pool that is actively proliferating (Fig. 2B and C). With LF diet, p was greater in male versus female mice (41.4% vs 37.2%; mean difference: 4.2 ± 1.0%). p was greater with HF diet in both female and male mice, with a larger effect size in female mice (mean difference: 10.4 ± 1.0%) versus male mice (mean difference: 7.8 ± 1.0%). Consequently, p was similar for female (47.6%) and male (49.3%) mice under HF diet conditions. Although HF diet caused a larger fraction of cells to proliferate, it decreased the rate of proliferation within the pool of actively proliferating cells, as indicated by a reduction in the synthesis rate value (k) (Fig. 2D). k was lower in both female and male mice with HF compared to LF diet, although the magnitude of the diet effect was greater in female mice (0.87/day vs. 0.36/day for LF vs. HF in females; 0.58/day vs. 0.40/day for LF vs. HF in males). Given that female mice had a greater proliferation rate than males under LF diet conditions (0.87/day vs. 0.58/day for females vs. males), the lower k in female mice resulted in similar proliferation rates in male and female mice under the HF diet condition (Fig. 2D). We then calculated k based on the total pool of cells, which includes proliferative and non-proliferative cells. When using this approach, the synthesis rate underestimates synthesis depending on how much of the pool is resistant to turnover, and the apparent inhibitory effect of HF diet is diminished (Fig. 2E). When we used k to estimate the half-life of actively proliferating cells, the half-life was approximately twice as long (2 days versus 1 day) with HF versus LF diet (Fig. 2F).

To complement the analysis of newly synthesized DNA, we measured RNA synthesis to determine ribosomal biogenesis. As with DNA, RNA was rapidly synthesized reaching a plateau value within 7 days of labeling (Fig. 2G). With LF diet, approximately half of the total RNA pool was actively renewing (Fig. 2H), which was slightly greater than the proportion of actively proliferating cells (Fig. 2B). Interestingly, whereas the percent of actively proliferating cells to total cells was greater with HF diet in both sexes, HF diet did not independently affect the percent of ribosomes undergoing biogenesis. Instead, there was a significant effect of sex ($p = 0.028$) and a significant sex-diet interaction ($p = 0.0005$). With LF diet, the proportion of actively synthesized pool of ribosomes was greater in female versus male mice (53.6% vs. 49.9%; $p = 0.0092$). The pool of actively synthesized RNA in male mice was greater with HF versus LF diet (55.5% vs 49.9%; $p = 0.0001$), whereas the active pool size did not change with HF diet in female mice ($p = 0.299$, Fig. 2H and I). Consequently, under HF diet conditions, male mice had a higher active pool size of newly synthesized ribosomes compared to female mice (55.5% vs. 52.2%, $p = 0.0131$) (Fig. 2H and I). Intriguingly, rates of ribosomal biogenesis (k) of the actively renewing pool were significantly altered by sex, HF diet, and their interaction (Fig. 2J). The rate of ribosomal biogenesis in female mice was more than twice as fast with HF versus LF diet (0.80/day vs. 0.37/day; $p < 0.0001$). In contrast, the rate of ribosomal biogenesis did not change between LF and HF diets in male mice. Consequently, with HF diet, ribosomes were synthesized at a significantly faster rate in female mice than in male mice ($p < 0.0001$) (Fig. 2J).

3.3. Sex-dependent effect of HF diet on targeted cartilage protein abundance

We next tested for sex differences in the effect of HF diet on the knee articular cartilage proteome. We quantified protein concentrations of specific extracellular matrix (ECM; Fig. 3), ECM-associated (Fig. 4), and cellular (Fig. 5) proteins through targeted high-resolution accurate mass measurements.

In the context of the ECM proteome, we assessed the concentrations of 7 collagen isoforms (Fig. 3A) and 9 proteoglycans (Fig. 3B). 5 of the 7 collagen isoforms were significantly altered by HF diet without any differences due to sex. HF diet resulted in greater concentrations of Col1a1, Col2a1, and Col11a1 compared to LF diet, whereas Col6a2 and Col6a3 concentrations were lower in HF versus LF diet (Fig. 3A). Among the proteoglycans, 6 of 9 proteins were significantly altered by HF diet (Fig. 3B). The concentrations of biglycan (Bgn), decorin (Dcn), fibromodulin (Fmod), lumican (Lum), and proline and arginine-rich end leucine-rich repeat protein (PreLP) were lower in the HF versus LF diet group, while proteoglycan 4 (Prg4) was greater in the HF versus LF diet group. Additionally, sex had a significant effect on the concentrations of Fmod, Dcn, and fibronectin-1 (Fn1), all being greater in male versus female mice (Fig. 3B).

HF diet and sex also altered the concentrations of numerous ECM-associated cartilage proteins (Fig. 4). The concentration of cartilage intermediate layer protein (Cilp), cartilage oligomeric matrix protein (Comp), Lamin A (Lmna), and Matrilin 1 (Matn1) were all lower under HF versus LF diet conditions. In contrast, the concentration of Milk fat globule EGF and factor V/VIII domain-containing protein (Mfge8) and secreted phosphoprotein 1 (Spp1; also known as osteopontin) were greater with HF versus LF diet. There were also numerous sex-dependent differences, with several proteins being more abundant in male mice (i.e., chitinase-like protein 3 (Chil3), clusterin (Clu), Cilp, and Lmna), and other proteins being more abundant in female mice (i.e., vitronectin (Vtn) and thrombospondin 1 (Thsb1)). Notably, Matrilin 3 (Matn3) exhibited a significant diet-sex interaction, being greater in female versus male mice under HF diet condition only (Fig. 4).

Finally, we assessed the concentrations of cellular proteins. Among proteins involved in cellular stress responses, peptidylprolyl isomerase A (Ppia) was greater with HF versus LF diet, whereas two heat shock proteins, Hsp90b and Hspa1a, were significantly lower under HF versus LF diet conditions (Fig. 5A). The concentration of Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was also lower with HF versus LF diet, particularly in male mice (Fig. 5B). Intriguingly, Solute carrier family 2 member 1 (Slc2a1; also known as glucose transporter 1), was significantly altered by HF diet, with trends for sex ($p = 0.0799$) and sex-diet interaction ($p = 0.0962$) effects. Post-hoc comparisons showed that

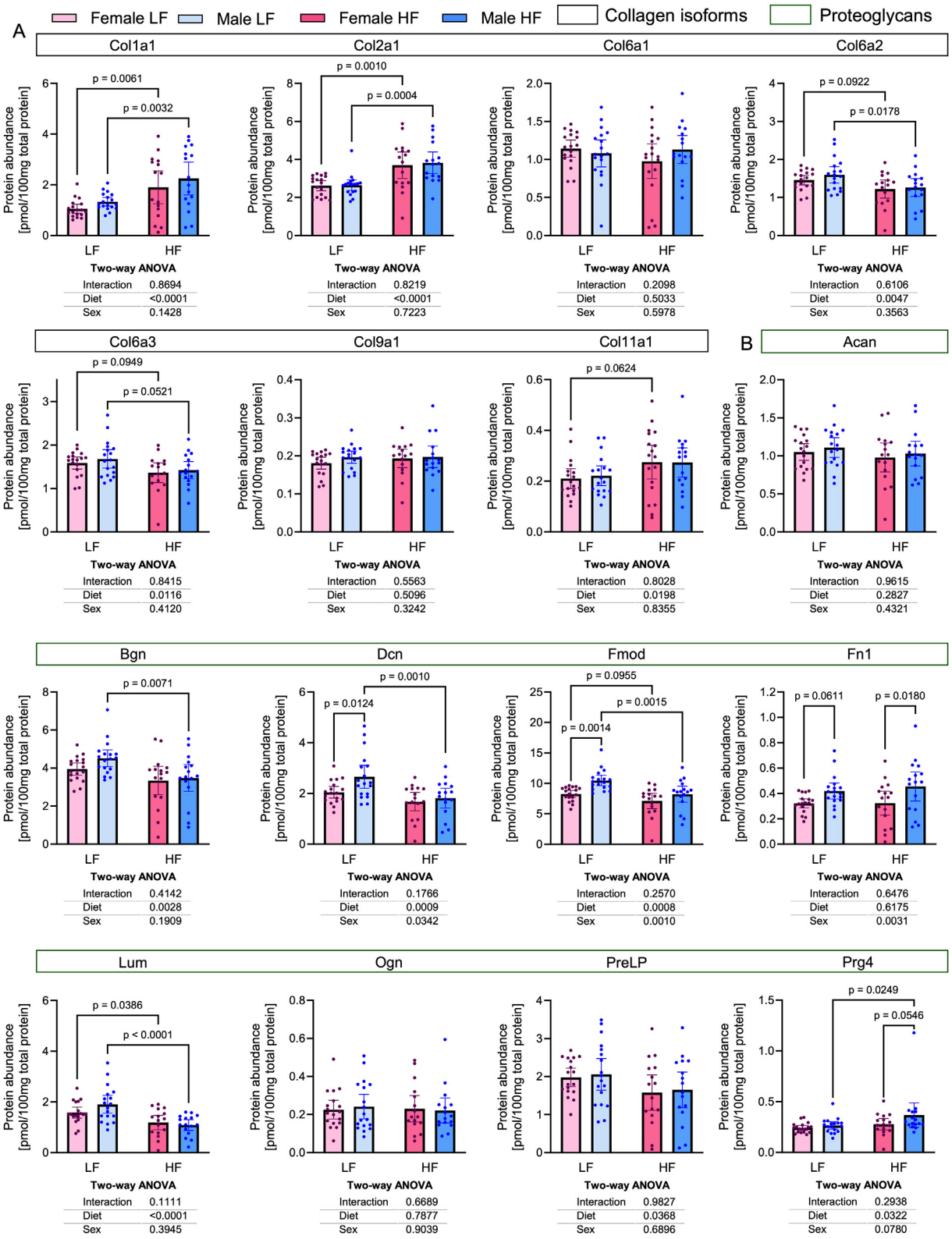

Fig. 3. Effect of 4-week LF and HF diet on extracellular matrix protein abundance in knee articular cartilage from male and female mice. (A) Protein abundance of collagen isoforms. (B) Protein abundance of proteoglycans. Each point is the protein concentration (pmol/100 mg total protein) obtained from tibial and femoral cartilage pooled from left and right knees of a single animal. Data are mean ± 95%CI. Effects of diet and sex evaluated using Two-way ANOVA, with Fisher LSD test used for post-hoc multiple comparisons ($p < 0.10$ shown). n = 16–18/per group.

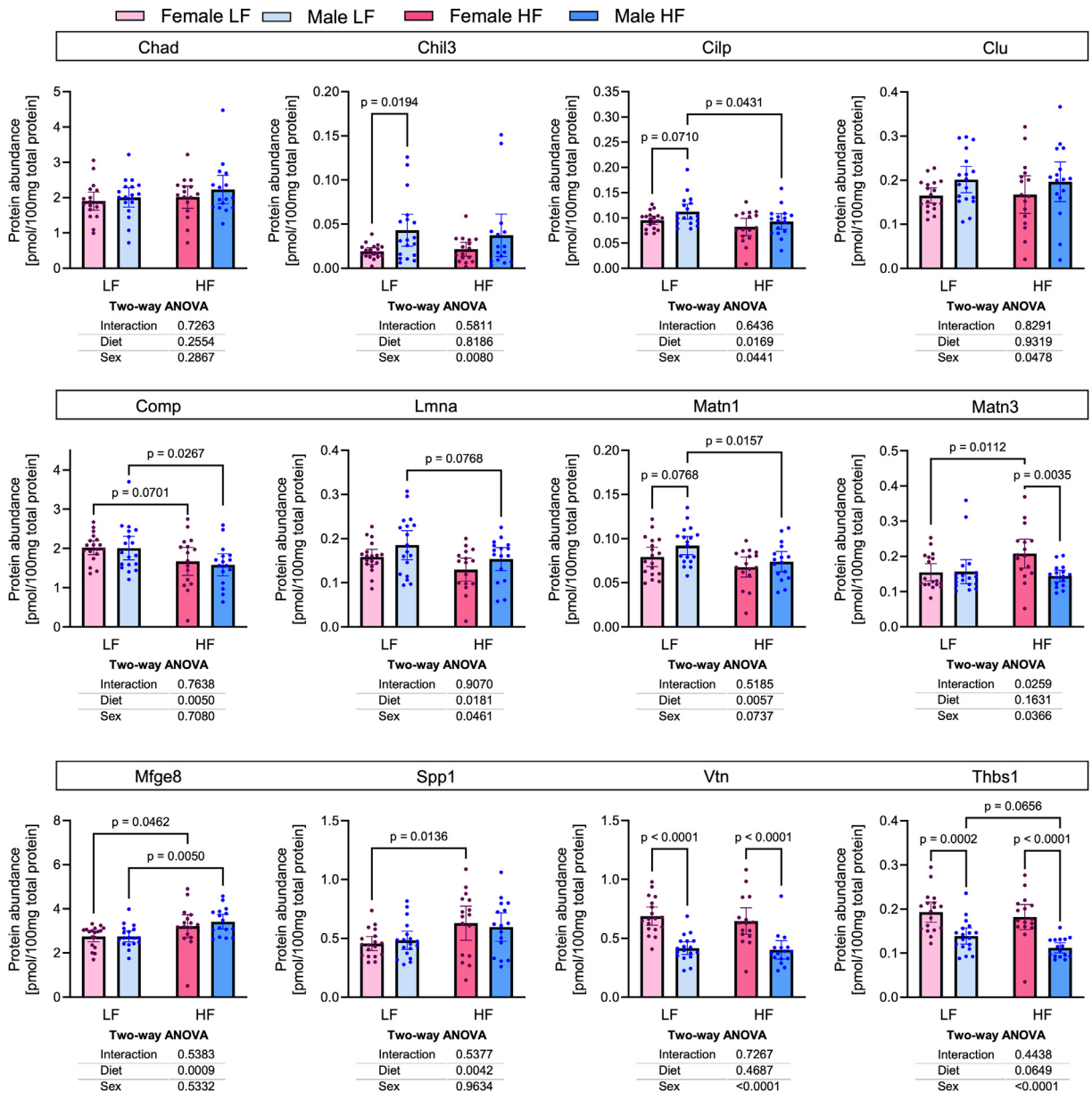

Fig. 4. Effect of 4-week LF and HF diet on the abundance of proteins associated with the extracellular matrix in knee articular cartilage from male and female mice. Each point is the protein concentration (pmol/100 mg total protein) obtained from tibial and femoral cartilage pooled from left and right knees of a single animal. Data are mean ± 95%CI. Effects of diet and sex evaluated using Two-way ANOVA, with Fisher LSD test used for post-hoc multiple comparisons ($p < 0.10$ shown). n = 16–18/per group.

Slc2a1 was greater with HF versus LF diet in male mice, resulting in greater Slc2a1 in male versus female mice with HF diet (Fig. 5B). Finally, no significant effects of HF diet or sex were observed among cytoskeletal proteins, although there was a trend for greater beta actin (Actbg) in the cartilage of male versus female mice (Fig. 5C).

Overall, HF diet treatment had a greater effect on cartilage protein abundance compared to sex-specific differences. Out of the 44 investigated proteins, 22 proteins significantly differed due to diet, whereas 10 proteins differed due to sex (Fig. 6A). A small number of proteins (Fmod, Dcn, Clip, Lmna) were sensitive to both diet and sex, and only one protein (Matn3) was altered by HF diet in a sex-dependent manner. Among the proteins altered by diet, most (14 of 22) were less abundant with HF diet (Fig. 6B). The most downregulated proteins included Gapdh and small leucine-rich proteoglycans, such as Fmod, Bgn, Dcn, and Lum. Conversely, the most upregulated proteins with HF diet included the RNA and peptide binding protein Ppia, fibrillar collagens (types I, II, and XI), and osteopontin (Spp1) (Fig. 6B). Among proteins with sex-dependent differences in abundance, most (7 of 10) were less abundant in female mice. For example, Chil3 and Fn1 were ~50% and 25% less abundant, respectively, in female versus male mice (Fig. 6B). In contrast, the adhesive glycoproteins vitronectin (Vtn) and thrombospondin 1 (Thbs1) were ~50–65% more abundant in cartilage from female versus male mice (Fig. 6B).

4. Discussion

We used *in vivo* deuterium oxide labeling and mass spectrometry to test the hypothesis that 4 weeks of HF diet treatment induces sex-specific changes in cellular mediators of cartilage homeostasis. By examining cell proliferation, ribosomal biogenesis, and protein abundance, we hoped to identify cell-intrinsic factors that contribute to sex-differences in OA risk. Overall, sex-dependent effects of HF diet treatment mostly involved cell

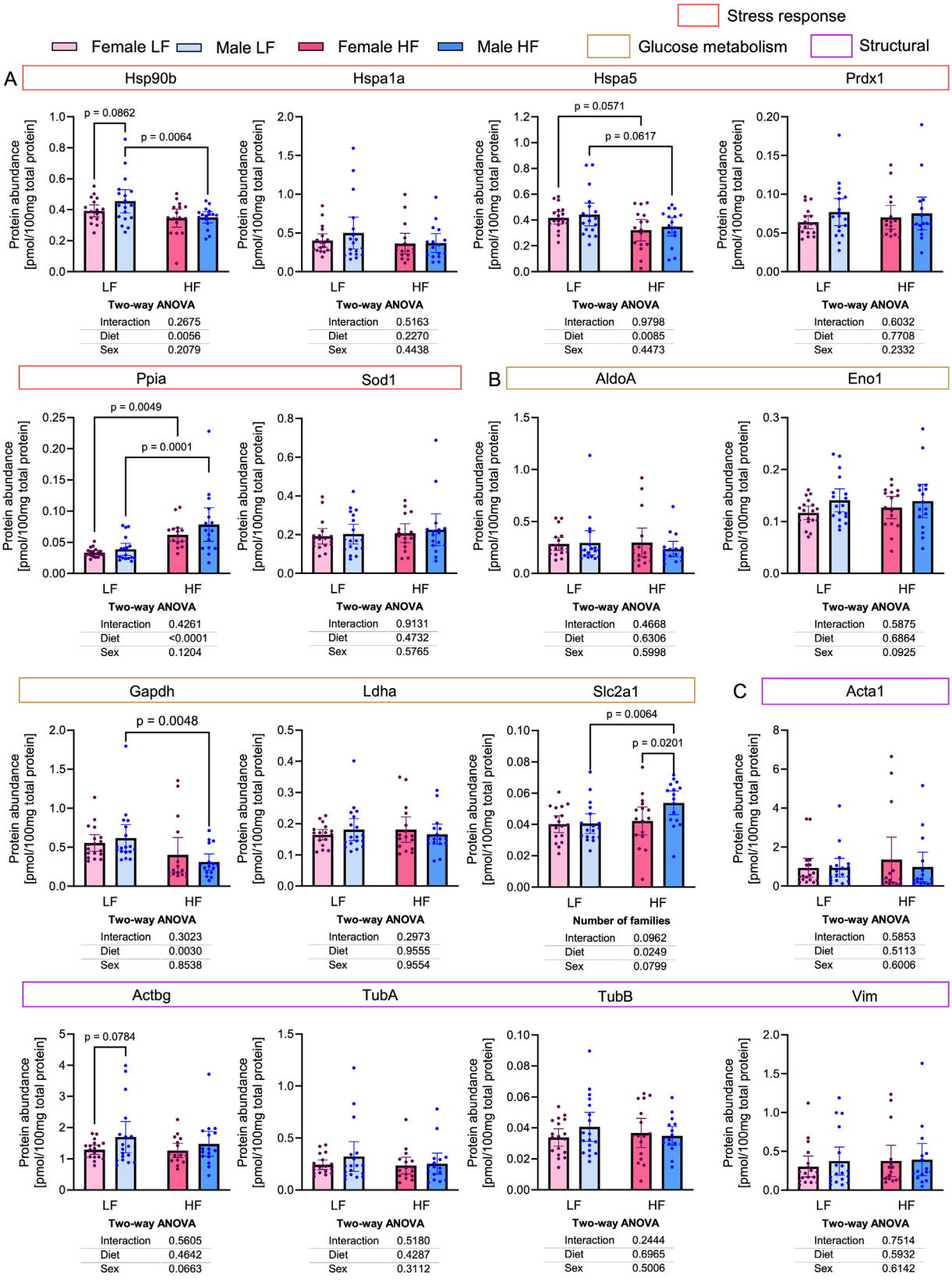

Fig. 5. Effect of 4-week LF and HF diet on cellular protein abundance in knee articular cartilage from male and female mice. Comparison of cellular proteins involved in (A) stress response, (B) glucose metabolism, and (C) cytoskeletal structure. Each point is the protein concentration (pmol/100 mg total protein) obtained from tibial and femoral cartilage pooled from left and right knees of a single animal. Data are mean ± 95%CI. Effects of diet and sex evaluated using Two-way ANOVA, with Fisher LSD test used for post-hoc multiple comparisons ($p < 0.10$ shown). n = 16–18/per group.

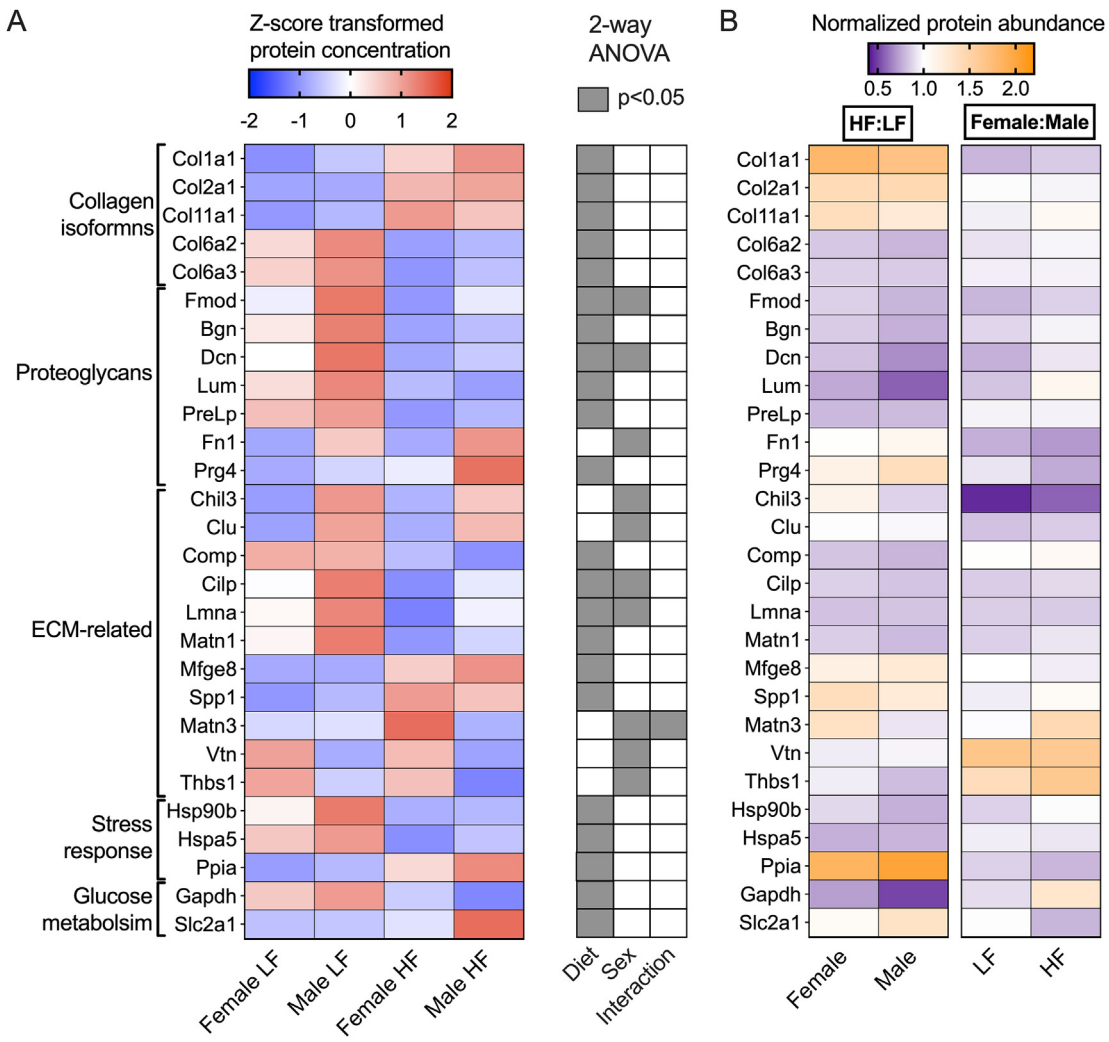

Fig. 6. Summary of HF diet and sex-dependent effects on the abundance of extracellular and cellular proteins in knee articular cartilage. (A) Heatmap of Z-score normalized protein concentrations from female and male mice treated with a LF or HF diet for 4 weeks. Only proteins with a significant ($p < 0.05$) effect of HF diet, sex-difference, or diet-sex interaction are shown, as determined by 2-way ANOVA. (B) Differences in cartilage protein concentration due to diet or sex were visualized by heatmap following normalization by diet (HF:LF) within females and males and normalization by sex (Female:Male) within LF and HF diet groups.

proliferation and ribosomal biogenesis. For cartilage cell proliferation, these effects involved sex differences in the magnitude rather than direction of change. For example, HF diet increased the fraction of the DNA pool that was renewing in both sexes, although the increase was a third greater in female mice. Similarly, HF diet treatment had lower DNA synthesis rates in both sexes (indicating longer cellular half-lives), although the effect was twice as great in female mice. For ribosomal biogenesis, HF diet caused distinct sex-dependent effects. In male mice, HF diet expanded the fraction of ribosomes renewing, whereas in female mice the rate of ribosome biogenesis was greater with HF diet. Surprisingly, just 4 weeks of HF diet induced changes in cartilage protein abundance characteristic of early OA, such as greater Col1a1 and Col2a1 and reduced proteoglycan content. Although these changes do not necessarily indicate the development of early-stage OA, the broad effect of 4-weeks HF diet treatment on cartilage cellular and ribosomal dynamics and protein abundance indicate the sensitivity of cartilage homeostasis to changes in diet.

Previous research on sex differences in chondrocyte proliferation has primarily focused on the regenerative potential of progenitor cell populations for promoting cartilage repair [28–30]. For example, male animals tend to have a higher proportion of progenitor cells with stronger chondrogenic potential than female animals, although proliferation rates are not intrinsically different between sexes [29,30]. Likewise, we observed that the proportion of proliferating cells in cartilage was greater in male versus female mice fed a LF diet. However, with HF diet, cellular proliferation increased more in female versus male mice such that the proportion of proliferating cells was similar in both sexes. Intriguingly, HF diet also increased the half-life of proliferating cells in both female and male mice, with a greater change observed in female mice. Although we do not know how an increase in both the relative proportion and half-life of proliferating cells are related, an analysis of progenitor cells in OA cartilage may provide some clues. Compared to healthy cartilage, OA cartilage from humans contains nearly twice as many chondrogenic progenitor cells, including a subpopulation of progenitor cells undergoing early senescence [31]. Could an increase in both the proportion and half-life of proliferating cartilage cells reflect the emergence of a HF diet-induced senescent progenitor cell subpopulation? Future research is needed to test this question, although a recent study reported that HF diet treatment in male mice induces chondrocyte senescence [32].

Additionally, we observed sex-related disparities in the effect of HF diet on rates of ribosomal biogenesis. In female mice, the rate of ribosomal biogenesis was twice as fast with HF diet treatment without a change in the fraction of the ribosomal pool that is renewing. In contrast, the fraction of the renewing ribosomal pool was greater with HF diet

treatment in male mice, without changes in the rate of biogenesis. Given that ribosomal biogenesis is a highly regulated process responsible for the translation of genetic information into proteins, these differences in ribosomal homeostasis may underlie important sex-dependent mechanisms of OA risk. Recent findings indicate that ribosomal dysfunction is a critical pathologic feature of OA [3]. However, more work is needed to understand how differences in ribosomal homeostasis and upstream regulators, such as mTOR [33], may contribute to sex differences in OA risk. Most effects of a HF diet on cartilage protein content were concordant between sexes, such as greater abundance of cyclophylin A (Ppia) and osteopontin (Spp1), two proteins previously associated with OA [34–36]. An exception was matrilin-3 (Matn3), which was more abundant in HF versus LF conditions in female mice only. Matn3 is a multifunctional ECM adaptor protein that exerts context-dependent anabolic and catabolic activities in cartilage [37]. Extended HF diet treatment may result in more differentially regulated proteins in male and female mice.

A limitation of the current study is that it did not include a separate cohort of animals for histological analysis. Although negligible OA pathology is expected for 4-weeks HF diet treatment based on prior studies [12,15,16,38], direct diet and sex histological comparisons would aid with interpreting our results as most prior studies only involved male mice. It is not known if HF diet-induced obese mice recapitulate the finding in humans that obesity has a greater effect on OA risk in women versus men. Therefore, extrapolating findings to human populations should be done with caution. Finally, while the current study identified novel sex-dependent effects of HF diet on cartilage cell proliferation, ribosomal biogenesis, and targeted extra-cellular and cellular protein abundance, future studies are required to test how these changes relate to sex differences in OA risk, such as those identified for age-associated and post-traumatic OA outcomes [18,39,40].

In conclusion, we found that short-term HF diet treatment altered cartilage matrix protein abundance in a similar manner in male and female mice. These similar protein changes occurred despite sex differences in the effect of HF diet on cartilage cell proliferation and ribosomal biogenesis, suggesting that distinct sex-dependent cellular processes contribute to cartilage homeostasis.

Author contributions

Concept and design: KAK, AB, MTK, BFM, TMG; acquisition, analysis, and interpretation of data: KAK, AB, AJ, FFP, MTK, BFM, TMG; drafting and critical revision of article: KAK, AB, AJ, FFP, MTK, BFM, TMG; final approval of article: KAK, AB, AJ, FFP, MTK, BFM, TMG.

Data sharing

Data that support the findings of this study are available from the corresponding authors upon reasonable request.

Declaration of competing interest

The authors have no conflicts of interest to declare.

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